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Translational repression by the oocyte-specific protein P100 in *Xenopus*

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ABSTRACT

The translational regulation of maternal mRNAs is one of the most important steps in the control of temporal–spatial gene expression during oocyte maturation and early embryogenesis in various species. Recently, it has become clear that protein components of mRNPs play essential roles in the translational regulation of maternal mRNAs. In the present study, we investigated the function of P100 in *Xenopus* oocytes. P100 exhibits sequence conservation with budding yeast Pat1 and is likely the orthologue of human Pat1a (also called PatL2). P100 is maternally expressed in immature oocytes, but disappears during oocyte maturation. In oocytes, P100 is an RNA binding component of ribosome-free mRNPs, associating with other mRNP components such as Xp54, xRAP55 and CPEB. Translational repression by overexpression of P100 occurred when reporter mRNAs were injected into oocytes. Intriguingly, we found that when P100 was overexpressed in the oocytes, the kinetics of oocyte maturation was considerably retarded. In addition, overexpression of P100 in oocytes significantly affected the accumulation of c-Mos and cyclin B1 during oocyte maturation. These results suggest that P100 plays a role in regulating the translation of specific maternal mRNAs required for the progression of *Xenopus* oocyte maturation.

Introduction

Translational control of mRNAs is a primary regulatory step of gene expression during animal early development (Davidson, 1986). Protein synthesis depends on maternally inherited and stored mRNAs, mainly because transcription is highly restricted due to chromatin condensation in two consecutive M-phases during oocyte maturation and the necessity for rapid cell cycle progression during early embryogenesis. *Xenopus laevis* has long served as a good model to study the translational repression (masking) and subsequent activation (unmasking) of maternal mRNAs. A large pool of mRNAs is sequestered in the oocyte cytoplasm as stored mRNPs that are biochemically fractionated as ribosome-free mRNPs (Darnbrough and Ford, 1981; Tafuri and Wolffe, 1993).

The precise timing of the translational activation of each mRNA stored in oocytes is mainly mediated by specific cis-elements, most of which reside in the 3′ untranslated regions (UTRs) of mRNAs, and their cognate binding proteins (and possibly microRNAs) (Mowry and Cote, 1999; Colegrove-Otero et al., 2005). When *Xenopus* oocytes are induced to mature by progesterone, translational activation of *c-mos* mRNA, which encodes a MAP kinase kinase kinase, and subsequent MAP kinase signaling are prerequisites for the cytoplasmic polyadenylation and translational activation of *cyclin B1* mRNA (Ballantyne et al., 1997; de Moor and Richter, 1997; Radford et al., 2008). Both *c-mos* and *cyclin B1* mRNAs contain cis-elements called cytoplasmic polyadenylation elements (CPEs) (Fox et al., 1989; McGrew et al., 1989; McGrew and Richter, 1990; Sheets et al., 1994; Richter, 2007). Their cognate binding protein CPEB has been well-characterized to be required both for translational repression in immature oocytes and for cytoplasmic polyadenylation-dependent translational activation in maturing oocytes (Hake and Richter, 1994; Stebbins-Boaz et al., 1996; de Moor and Richter, 1999; Richter, 2007). More recently, the early translational activation of *c-mos* mRNA has been shown to be dependent on Musashi, which binds to a polyadenylation response element (PRE) in the *c-mos* 3′UTR (Charlesworth et al., 2002, 2006), whereas Pumilio is involved in the timing of the translational activation of *cyclin B1* mRNA (Nakahata et al., 2001, 2003).

The translational efficiency of an mRNA is also regulated by a set of proteins associating with it. Protein components of cytoplasmic mRNPs in *Xenopus* oocytes have been biochemically identified by sucrose gradient centrifugation and oligo(dT) cellulose chromatography. In previtellogenic oocytes, four abundant (50, 52, 56 and 59 kDa, named mRNP1–4, respectively) and less abundant (75, ~100, 16 and 22 kDa, named mRNP5–8, respectively) proteins are co-fractionated with polyA$^+$ RNAs as free mRNPs (Darnbrough and Ford, 1981). Subsequently, five proteins

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(p54, p56, p60, p70 and p100) have been found to selectively bind to mRNA (Murray et al., 1991). mRNP3 and mRNP4, which are identical to p54 and p56, respectively, are members of the Y-box protein family, with mRNP4 being identical to the Y-box protein FRGY2 (Tafuri and Wolffe, 1990; Deschamps et al., 1992; Murray et al., 1992). Y-box proteins are core components of mRNPs in higher eukaryotes, and likely responsible for the packaging of mRNAs into mRNPs (Matsumoto and Wolffe, 1998; Matsumoto et al., 2003; Skabkin et al., 2004). The addition of a sufficiently excess of Y-box proteins into *in vitro* translation systems or over-expression in cultured cells leads to the translational repression of reporter mRNAs (Matsumoto et al., 1996; Davydova et al., 1997; Tanaka et al., 2004). Later studies identified other components of mRNPs including the DEAD-box ATPase Xp54, the embryonic poly(A) binding proteins ePAB and ePABP2, xRAP55, 4E-T and the oocyte-specific eIF4E1b that binds to the cap weakly (Ladomery et al., 1997; Voeltz et al., 2001; Good et al., 2004; Cosson et al., 2004; Tanaka et al., 2006; Minshall et al., 2007). Importantly, Xp54, RAP55 and 4E-T were shown to repress translation *in vitro* and/or in oocytes, whereas ePAB stimulates translation (Minshall et al., 2001; Tanaka et al., 2006; Wilkie et al., 2005; Minshall et al., 2007). The Y-box protein, the homologues of Xp54, and RAP55 possibly constitute the abundant and conserved components of maternal mRNPs of different organisms (Audhya et al., 2005; Boag et al., 2005; Wilhelm et al., 2005; Tanaka et al., 2006). Their involvement in translational repression in a wide range of organisms is further supported by the fact that they are found in processing bodies (P-bodies) and stress granules, discrete cytoplasmic foci where non-translating mRNAs accumulate (Cougot et al., 2004; Tanaka et al., 2006; Yang et al., 2006; Suzuki et al., 2009).

We previously isolated FRGY2-containing complexes from *Xenopus* oocytes and identified xRAP55 in these complexes (Tanaka et al., 2006). We hereafter refer to this protein as xRAP55A, since recently xRAP55B was identified in small oocytes (Minshall et al., 2007; Marnef et al., 2009). In the same complexes, we identified P100, which was originally purified from *Xenopus* oocytes as a protein recognized by scleroderma patient sera (Tanaka et al., 2006; Rother et al., 1992). Although P100 was shown to bind to single-stranded DNA, it has not been characterized in detail. P100 exhibits sequence conservation with budding yeast Pat1p. Pat1p is involved in mRNA degradation by activating mRNA decapping, functioning together with the Lsm1p-7p heptameric complex (Bonnerot et al., 2000; Bouveret et al., 2000; Tharun et al., 2000). Pat1p also acts as a general translational repressor in yeast (Coller and Parker, 2005), although an earlier report has shown that Pat1 is involved in translation initiation (Wyers et al., 2000). In yeast, Dhh1p, the orthologue of Xp54, is also required for global translational repression of mRNAs, targeting mRNAs for decapping, and promoting their assembly into P-bodies (Coller and Parker, 2005). It has been shown that yeast Lsm1p-7p, Pat1p and Dhh1p form a complex to enhance the decapping (Coller and Parker, 2004).

In this study, we examined the function of P100 in *Xenopus* oocytes. We found that P100 was already present in the smallest oocytes and disappeared during oocyte maturation. In oocytes, P100 is an RNA binding component of ribosome-free mRNPs. Furthermore, overexpression of P100 repressed the translation of reporter mRNAs. We also found that when P100 was overexpressed by microinjecting *P100* mRNA, the kinetics of oocyte maturation was considerably retarded. Overexpression of P100 significantly affected the accumulation of c-Mos and cyclin B1 both of which are normally synthesized during oocyte maturation. Our data suggest that P100 is involved in the translation of maternal mRNAs required for the progression of oocyte maturation.

Materials and methods

Oocyte preparation and microinjection

X. laevis oocytes were obtained by surgical removal of ovaries from mature female frogs and treatment with collagenase. Stage VI oocytes were microinjected with 30–45 ng of *in vitro* transcribed mRNA or 20 ng of morpholino antisense oligonucleotides (MO, Gene Tools) and maintained at 18 °C for 12–15 h (Matsumoto et al., 1998). MO used were: P100, 5′-GTTCGGAGCCGAGATTCATGTTTGC-3′ and control, 5′-CCTCTTACCTCAGTTACAATTTATA-3′. Maturation was induced by incubating the oocytes in modified Barth's solution (MBSH) containing progesterone (5 μM). The oocytes were collected at appropriate intervals and scored for GVBD to examine the effects of mRNAs on oocyte maturation. Oocyte lysates were prepared as described previously (Matsumoto et al., 1999). Lysates from stage VI and matured oocytes were treated at 30 °C for 30 min with lambda protein phosphatase (New England Biolabs) in a buffer supplied by the manufacturer in the presence of Complete EDTA-free protease inhibitor cocktail (Roche) (Suzuki et al., 2009).

Antibodies and immunoblotting

Immunoblotting was performed as described previously (Matsumoto et al., 1999). Rabbit polyclonal antibodies against P100 and xPat1b were raised against synthetic peptides, EDKMECPVIPPYTAVPS, corresponding to the C-terminal region of P100, and CQFRPDTTHLHPQHRRM, corresponding to an internal region of xPat1b, respectively (see Fig. S1). Antibodies against FRGY2 (Tafuri and Wolffe, 1992), xRAP55A (Tanaka et al., 2006), TAF-I (a gift from Dr. K. Nagata), cyclin B1 (a gift from Dr. J. L. Maller), Lsm1 (Abcam), c-Mos and Erk1 (Santa Cruz), β-actin (Sigma) and CPEB (IMGENEX) were used as primary antibodies.

Immunocytochemistry of Xenopus oocytes

Polyclonal antibodies against P100 were affinity-purified with an antigen peptide column. For immunocytological staining, oocytes were fixed with 100% methanol and embedded in Polyester Wax (BDH Chem., UK). Sectioned specimens were stained with primary and secondary antibodies as described previously (Tanaka et al., 2004). The primary antibodies were used at a final concentration of 2 μg/ml. The secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit IgG antibodies (2 μg/ml, Molecular Probes). The specimens were observed under a fluorescence microscope (Olympus, AX70).

Immunoprecipitation

Stage I–III oocytes (200 μl) were homogenized in 500 μl of buffer C (20 mM HEPES [pH 7.5], 2 mM $MgCl_2$, 1 mM dithiothreitol [DTT] and 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl, as described previously (Aoki et al., 2003). The lysates were cleared by centrifugation at 18,000×g at 4 °C for 10 min. The lysates were treated with 50 μg/ml RNase A at 37 °C for 10 min, where indicated. The oocyte lysates were incubated with affinity-purified anti-P100 antibodies bound to protein A-agarose or protein G-Sepharose in buffer C containing 100 mM KCl at 4 °C for 2–3 h. After extensive washing with buffer C containing 175 mM KCl, complexes containing P100 proteins were eluted with 700 μg/ml of the P100 antigen peptide in 20 mM HEPES [pH 7.5]–150 mM KCl at room temperature for 5 min. The eluates were analyzed in a SDS-polyacrylamide gel. Tandem mass spectrometric analyses of the tryptic peptides were carried out at the Research Resources Center at our institute. RNA was prepared from oocyte lysates and immunoprecipitates and used as templates for reverse transcriptase-mediated PCR (RT-PCR). Primers used were: c-mos, 5′-AGTGTCATTCTCTGATCGG-3′ and 5′-CATCCTATCAAAGGAAGAC-3′; cyclin B1, 5′-AGGAGACAATGTTCATGAC-3′ and 5′-ACGTAGTCCTTCACATGAG-3′; RINGO, 5′-GATGAGGTAATGGCGAAAG-3′ and 5′-GGCAAGTATTATTCTCCAC-3′; β-globin, 5′-AAGACTATTGGACAAGAGG-3′ and 5′-TATGTAGCTTAGAGACTCC-3′.

Sucrose gradient centrifugation

Sucrose gradient fractionation of oocyte lysates was performed essentially as described previously (Matsumoto et al., 2000; Tanaka et al., 2006). Twenty stage VI oocytes were homogenized in 200 µl of buffer A (20 mM Tris–HCl [pH 7.5], 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 10 µg/ml cycloheximide and 25 U/ml RNase inhibitor). The homogenate was centrifuged in a microtube at 18,000×g at 4 °C for 10 min. The supernatant was loaded on a 15–40% sucrose density gradient prepared in buffer A and centrifuged at 280,000×*g* in a Hitachi P50S2 rotor at 4 °C for 2 h. For the EDTA treatment, all the buffers contained 20 mM EDTA instead of $MgCl_2$ and cycloheximide.

Oligo(dT)-cellulose chromatography and UV-crosslinking assay

Oligo(dT)-cellulose chromatography following UV-crosslinking was performed as described previously (Tanaka et al., 2006) using 200 µl of stage I–III oocytes.

Constructs

cDNAs for P100 (EXL1051-4011859, accession number BI449406) and xPat1b (EXL1051-97080295, accession number BC108769) were obtained from Open Biosystems. To generate a construct for the N-terminal MS2-tagged *P100* mRNA (pMS2-P100), the P100 coding sequence was amplified by PCR from the P100 cDNA with a primer set, 5′-CTAGCTAGCATGAATCTCGGCTCCGAAC-3′ and 5′-CTAGCTAGCTGAAGGTACAGCTGTGTAC-3′. The PCR fragment was digested with NheI and cloned into pMSP-poly(A)$_{60}$ (Tanaka et al., 2006).

A construct for the C-terminal FLAG-tagged *P100* mRNA (pP100-FLAG-C) was generated as follows. Two oligonucleotides, 5′-AGCTTGGAAGATCTTCCCCCGGGGGTTACCGACTAGTGATTATAAAGATCATGACATCGACTACAAGGATGACGATGACAAGTGAC-3′ and 5′-AGCTGTCACTTGTCATCGTCATCCTTGTAGTCGATGTCATGATCTTTATAATCACTAGTCGGTAACCCCCGGGGGAAGATCTTCCA-3′, were annealed and cloned into the HindIII site of pGEM-pA+ (Aoki et al., 2003) to generate pGEM-FLAG encoding the 2xFLAG sequence. The P100 coding sequence was amplified by PCR from the P100 cDNA with a primer set, 5′-GAAGATCTCGCAAACATGGATCTCGG-3′ and 5′-GACTAGTTGAAGGTACAGCTGTGTAC-3′. The PCR fragment was then digested with BglII and SpeI and the resulting fragment was cloned into pGEM-FLAG to obtain pP100-FLAG-C.

To generate a construct for the N-terminal FLAG-tagged *P100* mRNA (pN-FLAG-P100), the P100 coding sequence was amplified by PCR from P100 cDNA with a primer set, 5′-GAAGATCTCGCAAACATGGATCTCGG-3′ and 5′-TCTAGAGGATCCTCATGAAGGTACAGCTGTG-3′ containing a termination codon. The PCR fragment was digested with BglII and BamHI and cloned into the p3xFLAG-CMV10 expression vector (Sigma) to obtain pCMV10-P100. The 3xFLAG-P100 coding sequence was amplified by PCR from pCMV10-P100 with a primer set, 5′-CTCTAGACTAGTATGGACTACAAAGACCATG-3′ and 5′-CTCTAGACTAGTTCATGAAGGTACAGCTGTG-3′. The PCR fragment was digested with SpeI and cloned into pGEM-FLAG to obtain pN-FLAG-P100. pMS2-P100, pP100-FLAG-C and pN-FLAG-P100 were linearized with NsiI and used for *in vitro* transcription with a mMESSAGE mMACHINE SP6 kit (Ambion).

To generate a construct for the *xPat1b* mRNA, the xPat1b coding sequence was amplified by PCR from xPat1b cDNA with a primer set, 5′-GGGAATTCCATATGGAGTCTCTAGTTGATGGTG-3′ and 5′-CCGCCGCTCGAGTAGTGTGCCGTTAGTTGATCCAAGC-3′. The PCR fragment was digested with NdeI and XhoI and cloned into pET24b vector (Novagen) to obtain pET-xPat1b. This plasmid was linearized with Bpu1102I and used for *in vitro* transcription with T7 RNA polymerase.

To generate a construct for the Rluc-β-globin reporter mRNA, a cDNA of *Renilla* luciferase was inserted into EcoRI–BamHI sites of pGEM11Zf(+) (Promega) to obtain pGEM-Rluc. The 3′UTR of *Xenopus β-globin* mRNA was obtained by RT-PCR from *Xenopus* oocyte RNA with a primer set, 5′-GAGCGCGGATCCACCAGCCTCAAGAACACC-3′ and 5′-ATGCATCAAGCTTAGAATGTGAAGAAACTTTC-3′. The PCR fragment was digested with BamHI and HindIII and cloned into pGEM-Rluc to obtain pGEM-Rluc-β-globin. Other constructs were described in Tanaka et al. (2006) (Tanaka et al., 2006).

Tethering assay

The mRNAs (30–40 ng) encoding MS2 and MS2-P100 were injected into stage VI oocytes. After 6 h, the oocytes were injected with 1.5–2 ng of luciferase reporter mRNA. Oocytes were then homogenized in 20 µl per oocyte of protein homogenization buffer (90 mM Hepes pH7.5, 70 mM KCl, 5% sucrose and 1 mM DTT) and 5-µl and 15-µl samples were assayed for firefly luciferase activity. To analyze mRNA stability, [^{32}P]-labeled mRNAs were injected as above and RNA was recovered with TRI reagent (Sigma) and analyzed by electrophoresis in an agarose gel containing formaldehyde. The gels were dried and exposed to an imaging plate. The radioactivity was quantified using a BAS2500 Image Analyzer (Fuji Film).

Analysis of protein synthesis activity

To analyze the protein synthesis in oocytes injected with *P100-FLAG-C*, *MS2* or *MS2-P100* mRNA, oocytes were incubated with [^{35}S] methionine and cysteine (100 µCi/ml, GE Healthcare) for 16–19 h. Oocytes were then homogenized in the protein homogenization buffer and analyzed by SDS-PAGE. The gels were dried and exposed to an imaging plate. The radioactivity of the translational products was quantified using a BAS2500 Image Analyzer.

Real time PCR

RNA was isolated from stage VI oocytes and matured oocytes with TRI reagent and cDNA was obtained using SuperScript III first strand synthesis system (Invitrogen). The amount of individual mRNA was determined by quantitative PCR using SYBR Premix EX Taq II and Thermal Cycler Dice real time system (Takara). The primers used were: ornithine decarboxylase (ODC), 5′-GCCATTGTGAAGACTCTCTCCATTC-3′ and 5′-TTCGGGTGATTCCTTGCCAC-3′ (Arthur et al., 2009); cyclin B1, 5′-GGATGCACAAGCAGTCAGACAAA-3′ and 5′-ACCTGGACCAGCCAGTCAATCA-3′; c-mos, 5′-TGGCGCTGAAGAAGGTAAAACG-3′ and 5′-GTTCAGTTCGGCCCAGAAGCT-3′.

Histone H1 kinase assay

H1 kinase assays were performed essentially as described (Walter et al., 1997; Iwabuchi et al., 2000). Oocytes were homogenized in 20 µl/oocyte of kinase buffer (80 mM β-glycerophosphate, 20 mM EGTA, 5 mM $MgCl_2$, 20 mM Hepes pH7.5, 1 mM DTT, 0.1% NP-40 and 1 × Complete EDTA-free protease inhibitor cocktail (Roche)) and cleared by centrifugation at 18,000×g at 4 °C for 10 min. Oocyte lysates equivalent to half an oocyte were mixed with 10 µl of a mixture containing 2.5 µg of histone H1 (Roche), 0.4 mM ATP, 1 µCi [γ-^{32}P]ATP, 20 µM cAMP-dependent kinase inhibitor (Promega) and 200 µg/ml bovine serum albumin, and incubated at 30 °C for 10 min. The reactions were stopped by adding SDS-PAGE sample buffer. Proteins were analyzed by SDS-PAGE.

Results

Temporal and spatial expression of P100

We previously identified P100 as a common component of FRGY2- and xRAP55A-containing RNPs isolated from *Xenopus* oocytes (Tanaka et al., 2006). P100 has sequence conservation with budding yeast Pat1p. Recently, two Pat1p homologues in mammals, Pat1b/PatL1 and Pat1a/PatL2, were identified (Scheller et al., 2007). *Pat1b*/*PatL1* mRNA is expressed ubiquitously in humans and mice, while *Pat1a*/*PatL2*

expression is restricted to the ovary, extraembryonic tissue and testis in mice ((Scheller et al., 2007); NCBI UniGene's EST ProfileViewer at http://www.ncbi.nim.nih.gov/UniGene). *Xenopus* P100 is expressed only in the ovary (Rother et al., 1992) and has a higher degree of sequence conservation with Pat1a/PatL2 than with Pat1b/PatL1 (Fig. 1A). A database search led to the identification of *Xenopus* Pat1b (xPat1b) (Figs. 1A and S1). Thus, there seems to be two Pat1 homologues in metazoans, one expressed ubiquitously and the other expressed in reproductive tissues.

To facilitate characterization of xPat1a/P100, we have raised a polyclonal antibody against a synthetic peptide corresponding to the P100 C-terminus. This antibody reacted with a ~100 kDa protein in oocyte lysates (Fig. 1B), as well as with a recombinant P100 expressed in oocytes (see Fig. 5B). Using this antibody, we examined the expression of P100 in oocytes, eggs and early embryos (Fig. 1C). P100 was already expressed in the smallest stage I oocytes and the amount of P100 decreased during oocyte growth until stage VI, essentially as reported (Rother et al., 1992; Minshall et al., 2007). These expression patterns are in contrast to those of xRAP55A (Fig. 1C, Tanaka et al., 2006) and FRGY2 (data not shown), both of which increase during oogenesis. Strikingly, P100 became undetectable in unfertilized and fertilized eggs and remained absent in early embryos until the tailbud stage (Fig. 1C). These results raise the possibility that P100 disappears during oocyte maturation. To examine this, we treated stage VI oocytes with progesterone to induce maturation. The immature oocytes that have been arrested at prophase of meiosis I resume meiosis under the control of progesterone and become arrested at metaphase of meiosis II at the end of oocyte maturation. The resumption of meiosis is characterized visually by the appearance of a white spot on the animal pole of the oocyte, which is the consequence of germinal vesicle breakdown (GVBD). When GVBD occurred in 50% of the progesterone-treated oocytes, each oocyte without or with a white spot was individually lysed and assayed for P100 by immunoblotting. We found that the amount of P100 was significantly smaller in the oocytes with white spots than those without white spots, while the amount of xRAP55A did not change appreciably (Fig. 1D). These results imply that P100 decreases depending on the progression of oocyte maturation. This notion was further supported by a time-course experiment showing that the amount of P100 rapidly decreased in pools of oocytes collected immediately after GVBD and thereafter at 1-h intervals (Fig. 1E).

We also raised a polyclonal antibody against a synthetic peptide corresponding to an internal region of xPat1b. This antibody detected putative endogenous xPat1b, which co-migrated with overexpressed recombinant xPat1b in stage VI oocytes (Fig. S2A). This antibody also reacted with P100, probably because the similarity between amino acid sequences of the antigen peptide and of the corresponding region in P100 (Fig. S1). xPat1b started to accumulate from stage IV oocytes and was expressed throughout early embryogenesis (Figs. S2B–D). This expression pattern of xPat1b is in sharp contrast to P100, which disappeared during oocyte maturation, suggesting a switchover from P100 to xPat1b. xPat1b was detected as a doublet in oocytes and only

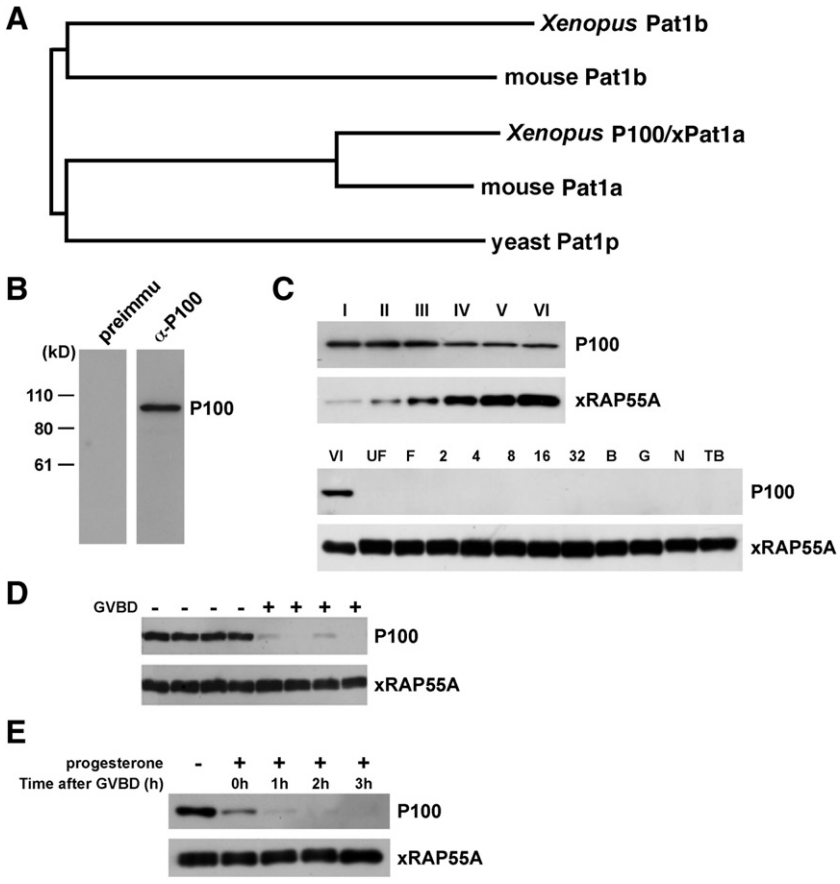

Fig. 1. Temporal expression of P100 protein. (A) Phylogenetic tree of Pat1 family members. *Xenopus* P100 (Genbank accession number NP_001085311) is more closely related to mouse Pat1a (AAI45647, ClustalW score 36.2) than to *Xenopus* Pat1b (AAI08770, score 23.0), mouse Pat1b (AAH58941, score 25.0) and yeast Pat1p (CAC42990, score 11.9). (B) Immunoblotting of lysates from stage VI oocytes with anti-P100 antibody or preimmune serum. (C) Temporal expression of P100 during oogenesis (upper panels) and early embryogenesis (bottom panels). Lysates equivalent to 0.5 oocytes (stages I through VI), unfertilized egg (UF), fertilized egg (F), or embryos at the 2-cell, 4-cell, 8-cell, 16-cell and 32-cell stages, blastula (B), gastrula (G), neurula (N) and tailbud (TB) were analyzed by immunoblotting. (D) Disappearance of P100 in matured oocytes. Stage VI oocytes were stimulated with progesterone to mature for 8 h. Individual oocytes without (−) or with (+) a white spot, indicative of GVBD, were lysed and analyzed by immunoblotting with anti-P100 and anti-xRAP55A antibodies. (E) The gradual disappearance of P100 in matured oocytes. The stage VI oocytes were incubated in the absence (−) or presence (+) of progesterone. Oocytes were collected just after the appearance of white spots (0 h), or at the indicated time points after the appearance of white spots. Proteins were analyzed by immunoblotting with anti-P100 and anti-xRAP55A antibodies.

the slow migrating form was present in matured oocytes and eggs (Figs. S2C and D). This represents phosphorylation, because the slow migrating form was disappeared by phosphatase treatment (Fig. S2E).

P100 was predominantly located in the oocyte cytoplasm, as judged by immunoblotting of the nuclear and cytoplasmic fractions of stage VI oocytes (Fig. 2A). As a control, template activating factor I (TAF-I), which is involved in chromatin remodeling, was exclusively detected in the nuclear fractions (Matsumoto et al., 1999). The cytoplasmic localization of P100 was confirmed by the immunostaining of oocytes (Fig. 2B). P100 was distributed evenly in the oocyte cytoplasm throughout oogenesis. Although the mRNP component xRAP55A was localized to cytoplasmic foci in stage VI oocytes (Tanaka et al., 2006), localized signals for P100 were not obvious.

P100 is an RNA binding component of stored mRNPs in oocytes

Given that P100 was coimmunoprecipitated with FRGY2 and xRAP55A (Tanaka et al., 2006), we examined whether P100 is a component of cytoplasmic mRNPs. Lysates from stage VI oocytes were fractionated on a sucrose gradient in the presence of cycloheximide and the distribution of P100 was examined. P100 was distributed predominantly in fractions lighter than ribosomes, corresponding to fractions containing non-translating (storage) mRNPs (Fig. 3A). FRGY2 was mainly detected in non-translating mRNP fractions with minor portions in mRNA-free fractions and ribosomal fractions, as reported previously (Tanaka et al., 2006). To verify our observation, oocyte lysates were treated with EDTA and fractionated on a sucrose gradient containing EDTA, under which conditions ribosomes and their subunits are released from mRNAs. The EDTA treatment did not change the distribution of P100 significantly, indicating that P100 predominantly exists in the ribosome-free mRNP fractions (Fig. 3B).

Because P100 was reported to bind to single-stranded DNA cellulose, it may bind to mRNA directly, as is also the case for most of the protein components of mRNPs identified to date (Colegrove-Otero et al., 2005; Matsumoto, 2006). Yeast Pat1p has been shown to bind to RNA (Pilkington and Parker, 2008). Alternatively, it is possible that P100 associates with mRNA indirectly through protein–protein interaction. To distinguish between these possibilities, we examined whether P100 is directly associated with adenylated RNAs in oocytes. To do this, oocyte lysates were irradiated to UV and subjected to oligo (dT)-cellulose chromatography. The oligo(dT)-cellulose was then washed with a buffer containing SDS, with which proteins not crosslinked to adenylated RNAs would have been washed away, and the bound fractions were analyzed after digestion with an RNase for the presence of P100. The immunoblotting revealed that P100 was retained on the oligo(dT)-cellulose depending on the UV-crosslinking as was FRGY2. In contrast, β-actin was not retained on the oligo(dT)-cellulose. These results demonstrate that P100 directly associate with adenylated RNA (Fig. 3C).

To identify which set of proteins are associated with P100 in oocytes, lysates from early stage oocytes (stages I–III), in which P100 is abundant (Fig. 1C), were used for immunoprecipitation with the anti-P100 antibody. When eluted by incubation with the antigen peptide, a number of proteins were specifically coimmunoprecipitated with P100 (Fig. 3D). The various bands were excised from the gel and proteins were identified by mass spectrometry. The identified proteins included 4E-T, ePAB, FRGY2, CPEB, xRAP55B and Xp54, all of which were known to be mRNP constituents in oocytes (Tanaka et al., 2006; Minshall et al., 2007). Among them, 4E-T, FRGY2, CPEB and

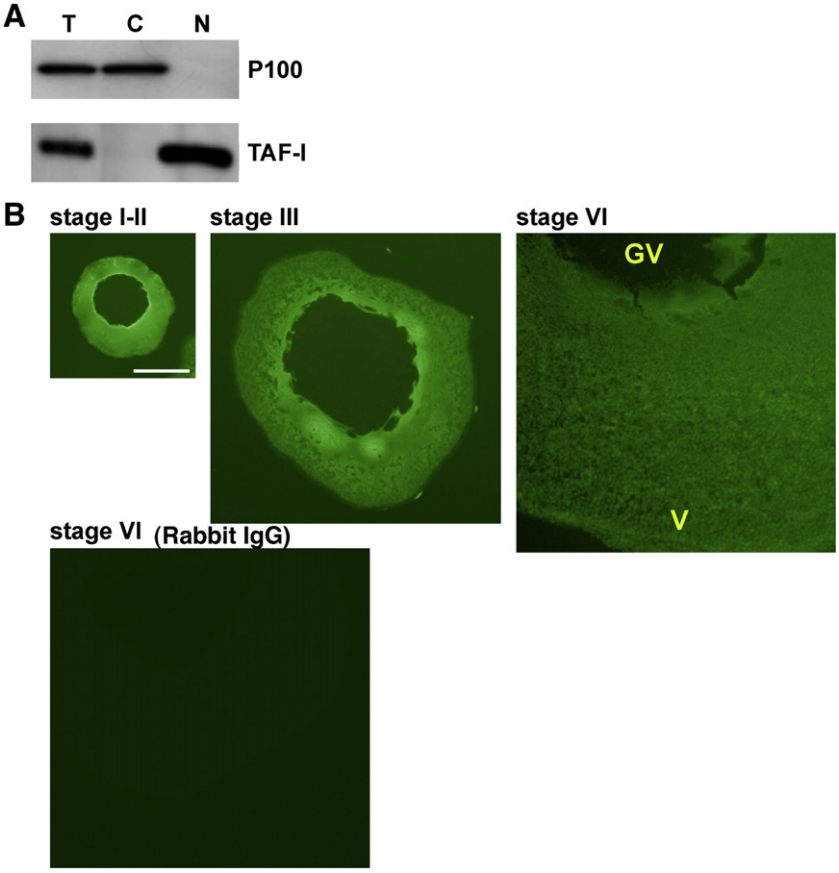

Fig. 2. Spatial expression of P100 protein. (A) Total lysate (T) and nuclear (N) and cytoplasmic (C) fractions of stage VI oocytes were analyzed by immunoblotting with anti-P100 and anti-TAF-I antibodies. (B) Sections of stage I–II, III, and VI oocytes were stained with anti-P100 antibody or rabbit IgG. The germinal vesicle (GV) and vegetal region (V) are indicated. Scale bar, 100 μm.

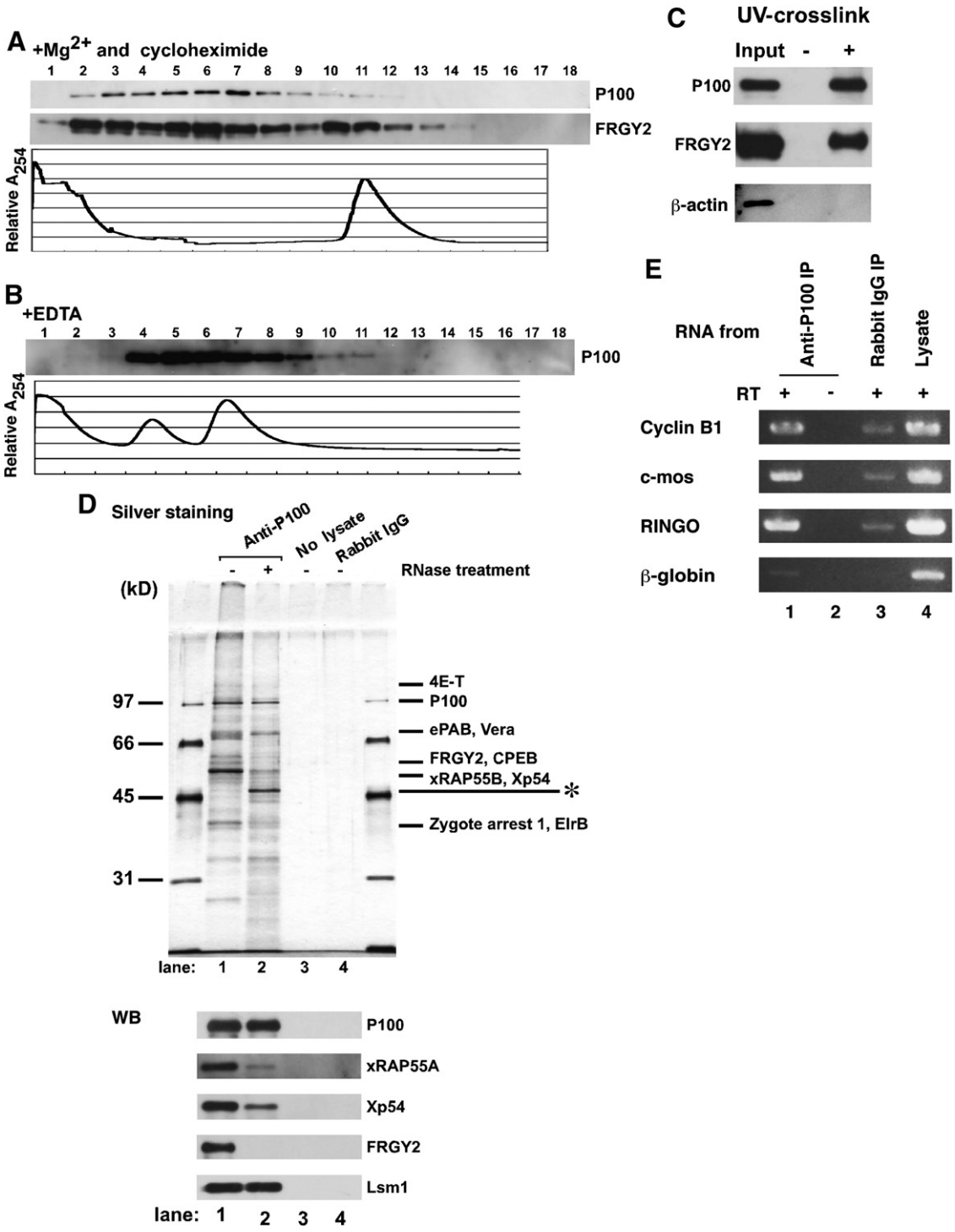

Fig. 3. P100 is an RNA binding component of cytoplasmic mRNPs. (A, B) Stage VI oocyte lysates were fractionated on a sucrose gradient in the presence of cycloheximide (A) or EDTA (B). Proteins from each fraction were analyzed by immunoblotting with anti-P100 and anti-FRGY2 antibodies. Absorbance profiles at 254 nm are shown. (C) Oocyte lysates were irradiated with UV light (lane 3) or not irradiated (lane 2) and subjected to oligo(dT) cellulose chromatography. After extensive washes with a buffer containing SDS, bound proteins were eluted and digested with RNase. Lysate (lane 1) and eluates (lanes 2 and 3) were analyzed by immunoblotting. (D) Isolation of a P100-associated complex by immunoprecipitation. Lysates were prepared from small oocytes (stages I–III). In lane 2, the lysates were treated with RNase. The lysates were incubated with anti-P100 antibody beads (lanes 1 and 2) or with rabbit IgG beads (lane 4). Anti-P100 antibody beads were incubated without lysate (lane 3). Bound proteins were resolved by SDS-PAGE and detected by silver staining (left). Relevant bands in lane 1 were analyzed by mass spectrometry, and proteins identified are listed on the right. A band corresponding to 48 kDa in lane 2 (indicated by an asterisk) was also analyzed and EF-1α was identified. P100, xRAP55A, Xp54, FRGY2 and Lsm1 in the immunoprecipitates were detected by immunoblotting (right). (E) Identification of P100-associating mRNAs. RNA was recovered from oocyte lysates and the immunoprecipitates either with anti-p100 antibody or rabbit IgG, and used as templates for RT-PCR.

Xp54 are involved in translational repression in oocytes. Furthermore, we identified the mRNA binding proteins Vera and ElrB, and zygote arrest 1 whose mouse homologue was shown to be a maternal effect gene (Wu et al., 2003). Elongation factor-1α (EF-1α), the most abundant protein in previtellogenic oocytes (Mattaj et al., 1987), was found as a major protein in the RNase-treated samples. We tried to confirm these results by immunoblotting of the immunoprecipitates with antibodies against mRNP components, and found that xRAP55A

in addition to FRGY2 and Xp54 was coimmunoprecipitated with P100. Moreover, we found that P100 was coimmunoprecipitated with Lsm1, as is the case for Pat1p in yeast (Bonnerot et al., 2000; Bouveret et al., 2000; Tharun et al., 2000). RNase-treatment prior to immunoprecipitation revealed that Lsm1, Xp54 and xRAP55A are associated with P100 in an at least partially RNA-independent manner. In contrast, FRGY2 was not coimmunoprecipitated with P100 from RNase-treated lysates. To examine whether mRNAs are associated with P100, RT-PCR was performed using RNA recovered from immunoprecipitates with anti-p100 antibody or with rabbit IgG (Fig. 3E). P100-interacting mRNAs included *cyclin B1*, *c-mos* and *RINGO* mRNAs. Taken together, these data further support the idea that P100 together with its associating proteins constitutes cytoplasmic mRNPs with maternal mRNAs in *Xenopus* oocytes.

P100 represses translation in oocytes

The above data revealed that P100 is an RNA binding component of mRNPs. Yeast Pat1p has been shown to be a general repressor of translation (Coller and Parker, 2005). We therefore investigated whether P100 plays a role in translational regulation. We first tested the effect of overexpression of P100 on global protein synthesis in stage VI oocytes. Oocytes were microinjected with an *in vitro* synthesized mRNA encoding C-terminally FLAG-tagged P100 (*P100-FLAG-C*) and incubated for 16–18 h to allow protein production. By incubating the oocytes with [^{35}S] methionine and cysteine and analyzing the newly synthesized proteins by SDS-PAGE, we found that overexpression of P100 did not have an apparent effect on global protein synthesis (Fig. 4A).

We then hypothesized that P100 would affect the translation of mRNAs when forced to bind to them. To test this, we tethered P100 to the 3′UTR of firefly luciferase (Luc) mRNA. mRNAs encoding either MS2 coat protein or MS2 fused to P100 were microinjected into oocytes (Fig. 4B). After incubation of the oocytes for 6 h to allow protein synthesis, capped luciferase mRNAs either containing MS2-binding sites (*Luc-MS2*) or not containing them (*Luc-ΔMS2*) were then injected. In contrast to no detectable effect on global translation, the expression of MS2-P100 repressed luciferase activity from *Luc-ΔMS2* mRNA to ~35% compared with that of MS2 protein alone (Fig. 4C). The luciferase activity from *Luc-MS2* mRNA was further repressed by MS2-P100 (20% of control). ^{32}P-labeled reporter mRNAs were equally stable in oocytes expressing MS2 or MS2-P100, suggesting the reduced luciferase activity to be due to differences in translation not in reporter mRNA stability. We conclude that P100 mediates the translational repression of the Luc reporter mRNA and this effect is augmented by tethering through MS2 and its binding sites. To examine whether the observed translational repression by P100 is specific for Luc mRNA, we used two additional reporter mRNAs that do not contain MS2-binding sites. Interestingly, when *chloramphenicol acetyltransferase (CAT)* mRNA was injected into MS2- or MS2-P100-expressing oocytes, virtually no translational repression was observed (Fig. 4D). In contrast, P100-FLAG-C repressed translation of *Renilla luciferase* mRNA connected to *Xenopus β-globin* 3′ UTR (Fig. 4E). These results suggest that P100 represses translation of a subset of mRNA.

Effect of P100 on the kinetics of meiotic maturation

After the triggering of meiotic maturation by progesterone, oocytes activate the translation of specific mRNAs by recruiting the mRNAs from storage mRNPs to ribosomes in an mRNA-specific manner depending on the progression of maturation. As P100 disappears during oocyte maturation, we investigated whether P100 has a role in the regulation of oocyte maturation by overexpressing *P100* mRNA. *P100-FLAG-C* mRNA was injected into stage VI oocytes and the oocytes were stimulated with progesterone to undergo meiotic maturation. The protein level of the exogenously expressed P100-FLAG-C was about the same as that of the endogenous P100 in immature oocytes. Oocytes overexpressing P100-FLAG-C underwent maturation very poorly, only ~40% of them showing GVBD 10 h after the progesterone treatment, at which time oocytes uninjected or injected with the *xRAP55A* mRNA underwent almost 100% maturation (Fig. 5A). When oocytes were injected with one-tenth the amount of *P100-FLAG-C* mRNA, only a slight retardation of oocyte maturation kinetics was observed, suggesting that the effect of P100 overexpression was dose-dependent. We noticed that, whereas the endogenous P100 in oocytes both uninjected and injected with *P100-FLAG-C* mRNA almost disappeared upon maturation, the protein level of the expressed P100-FLAG-C was not discernibly changed in matured oocytes compared to that in oocytes untreated with progesterone (Fig. 5B). To exclude the possibility that this behavior of P100-FLAG-C and its effect on oocyte maturation is due to the C-terminal FLAG-tag (i.e., the C-terminal tag might have disturbed the disappearance of P100-FLAG-C), we carried out a similar set of experiments with mRNA encoding N-terminally FLAG-tagged P100 (*N-FLAG-P100*). We observed no disappearance of N-FLAG-P100 during oocyte maturation (Fig. 5B) and a similar retardation of the maturation kinetics with oocytes expressing N-FLAG-P100 to that observed with P100-FLAG-C (Fig. 5A).

When oocytes were injected with one-tenth the amount of *P100-FLAG-C* mRNA and treated with progesterone, a small amount of P100-FLAG-C was expressed and both the endogenous P100 and the expressed P100-FLAG-C disappeared, suggesting that both the endogenous and the exogenous P100 are subjected to degradation (Fig. 6A). Furthermore, we examined the stability of P100-FLAG-C protein during maturation by injecting a morpholino antisense oligonucleotide (MO) (Fig. 6B). When an MO for P100 was injected into P100-FLAG-C-expressing oocytes before the addition of progesterone, the amount of P100-FLAG-C decreased almost as fast as the endogenous P100 during oocyte maturation. This effect was specific for P100 MO because a control MO did not affect the amount of P100-FLAG-C during maturation. In addition, both control and P100 MO had no significant effect on the disappearance of the endogenous P100. These experiments are consistent with the hypothesis that both the endogenous P100 and the FLAG-tagged P100 proteins are rapidly degraded after the addition of progesterone, but new FLAG-tagged P100 protein is continuously synthesized from the injected mRNA whereas the translation of the endogenous P100 mRNA would be somehow inhibited.

P100 overexpression inhibits the accumulation of c-Mos and cyclin B1 during oocyte maturation

Since we found that P100 overexpression represses mRNA translation and also affects the kinetics of oocyte maturation, we examined whether the accumulation of proteins that are normally synthesized during oocyte maturation would be repressed in P100-overexpressing oocytes. To this end, oocytes were microinjected with the *P100-FLAG-C* mRNA, treated with progesterone, and collected at 30-min intervals (Figs. 7A and B). The oocyte lysates were analyzed by western blotting. Whereas in control oocytes microinjected with water, c-Mos was well detected 4.5 h after the addition of progesterone, it was detected only 7 h after progesterone addition in P100 overexpressing oocytes. The accumulation of cyclin B1 was also occurred later in P100-overexpressing oocytes (9.5 h) than in water-injected oocytes (7.5 h). The activation of MAP kinase, revealed by phosphorylation of the enzyme, was also slower in P100-overexpressing oocytes (7.5 h) than in water-injected oocytes (5.5 h). To compare the levels of the accumulation of c-Mos and cyclin B1 between P100-overexpressing and control oocytes, the lysates of the same time points were analyzed in parallel (Fig. S3). Both c-Mos and cyclin B1 accumulated less in P100-overexpressing oocytes than in control oocytes, which was clearly evident in the lysates at 10 and 12 h after progesterone addition. The amount of *c-mos* and *cyclin B1* mRNAs in matured oocytes overexpressing P100 was not significantly different from that in matured control oocytes, as assessed by quantitative RT-PCR (Fig. S4), indicating that the P100

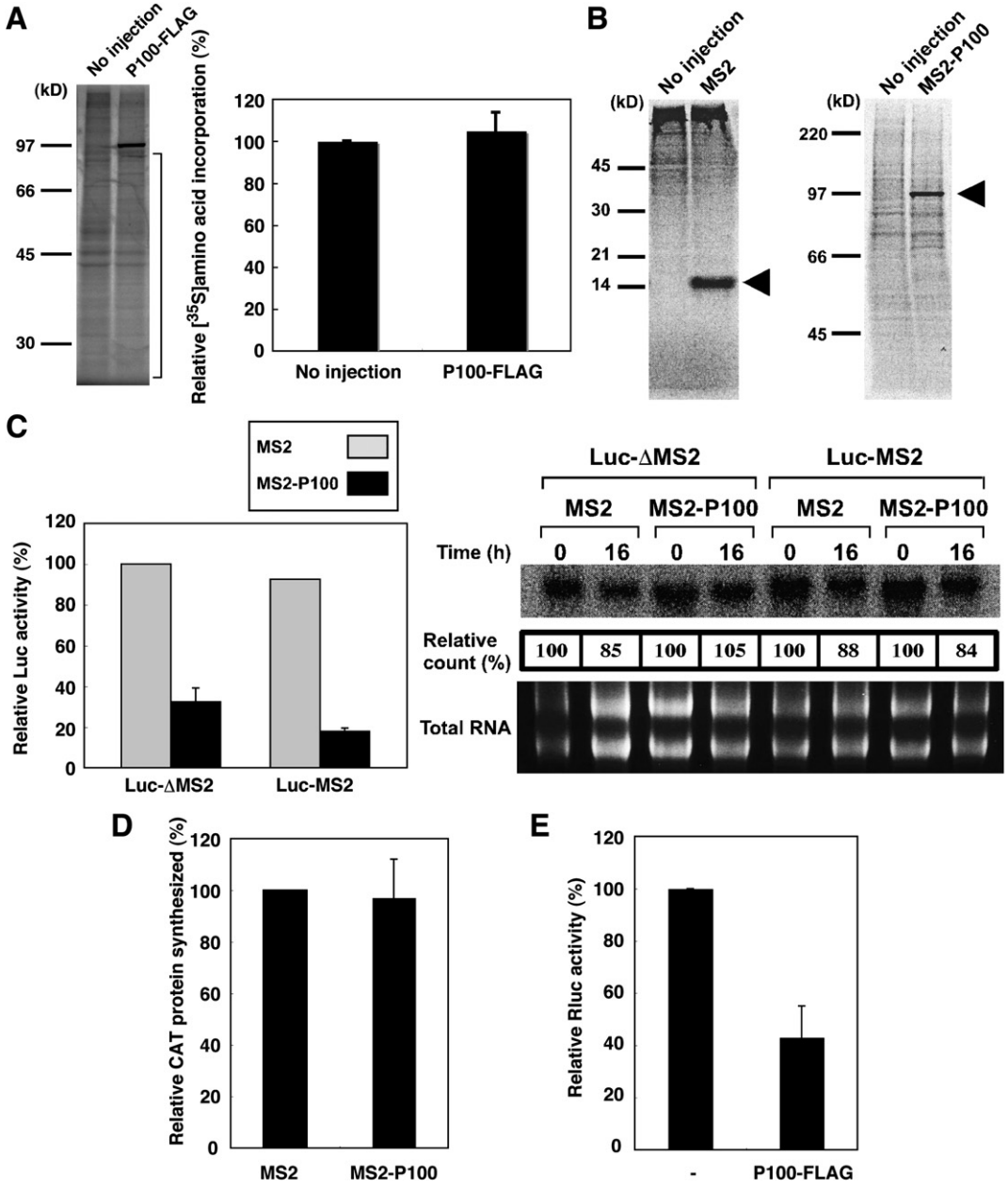

Fig. 4. Effect of P100 overexpression on protein synthesis in oocytes. (A) Analysis of protein synthesis in oocytes injected with *P100-FLAG-C* mRNA. Oocytes were incubated with [^{35}S] methionine and cysteine for 16 h and lysates of the oocytes were analyzed by SDS-PAGE. The radioactivity in the areas migrating faster than P100 (indicated by a bracket) was quantified. (B–C) Tethering assay. Oocytes were injected with mRNA encoding MS2 or MS2-P100. Expression of MS2 and MS2-P100 protein was analyzed by incubating the oocytes in [^{35}S] methionine and cysteine for 19 h (B). (C) Seven hours after the first injection, [^{32}P]-labeled and capped firefly luciferase reporter mRNAs either containing (Luc-MS2) or not containing (Luc-ΔMS2) MS2-binding sites were injected. After 17 h, oocytes were harvested and firefly luciferase activity was measured (left). Firefly luciferase activity in oocytes is shown relative to the case of Luc-ΔMS2-mRNA-injected oocytes expressing MS2 protein, which was set to 100%. The stability of [^{32}P]-labeled luciferase mRNAs in oocytes expressing MS2 and MS2-P100 was examined immediately after microinjection (0 h) and after 16 h of incubation (right). The relative count of [^{32}P]-labeled mRNA normalized to the value at 0 h, which was 100%, is shown in boxes. Total RNA served as recovery and loading controls. (D) Oocytes were injected with mRNA encoding MS2 or MS2-P100. Seven hours after the first injection, capped *CAT* mRNA was injected and expression of CAT protein was analyzed by incubating the oocytes in [^{35}S] methionine and cysteine. (E) Oocytes were injected with or without (−) *P100-FLAG-C* mRNA. Seven hours after the first injection, capped *Rluc-β-globin* mRNA was injected. After 17 h, oocytes were harvested and *Renilla* luciferase activity was measured.

overexpression did not affect the stability of these maternal mRNAs in immature and matured oocytes.

To investigate whether downstream events of oocyte maturation were also affected by the P100 overexpression, oocytes were collected at 1-h intervals after GVBD (Fig. 7C). In uninjected oocytes, c-Mos was easily detected at the time of GVBD and continued to accumulate during the maturation process. Cyclin B1 was detectable in stage VI oocytes and increased in its amount after GVBD. Cyclin B1 further accumulated at 2 to 3 h after GVBD, at which time control oocytes proceeded to meiosis II, as judged from histone H1 kinase activity. In P100-overexpressing oocytes, the protein levels of c-Mos and cyclin B1 at GVBD were initially the same as those in control oocytes. Although slightly less c-Mos accumulated in P100-overexpressing oocytes than in control oocytes, the accumulation of cyclin B1 at 2 to 3 h after GVBD was significantly impaired in P100-overexpressing oocytes. CPEB was degraded upon maturation, and the amount of xRAP55A remained constant as described previously (Hake and Richter, 1994; Tanaka et al., 2006). There was no striking difference between the amounts of these proteins in P100-overexpressing oocytes

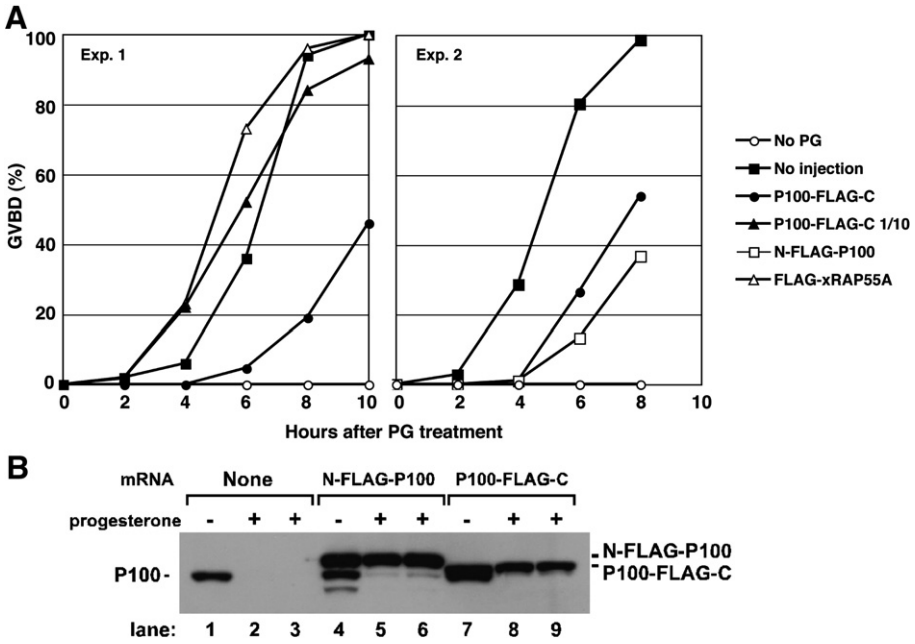

Fig. 5. Inhibition of maturation in oocytes overexpressing P100-FLAG-C. (A) P100 overexpression during oocyte maturation. Stage VI oocytes were injected with 45 ng of *P100-FLAG-C* mRNA (closed circles), 45 ng of *N-FLAG-P100* mRNA (open squares), 45 ng of *FLAG-xRAP55A* mRNA (open triangles), or 4.5 ng of *P100-FLAG-C* mRNA (closed triangles), or not injected (closed squares). They were incubated for 16–18 h, and then stimulated with progesterone (PG). Oocytes were scored as a fraction of time for GVBD. Two representative experiments are shown. At least 45 oocytes were analyzed for each condition in experiment 1, and at least 71 oocytes were analyzed in experiment 2. Oocytes incubated in MBSH without progesterone were not induced to mature at all (No PG, open circles). (B) Oocytes uninjected (lanes 1–3) or injected with 35 ng of *N-FLAG-P100* mRNA (lanes 4–6) or 35 ng of *P100-FLAG-C* mRNA (lanes 7–9) were induced to mature with progesterone (lanes 2, 3, 5, 6, 8 and 9). Lysates from stage VI and matured oocytes were analyzed by immunoblotting with anti-P100 antibody. Positions of endogenous P100, N-FLAG-P100 and P100-FLAG-C are indicated.

and those in uninjected controls. These results suggest that P100 regulates the translation of specific maternal mRNAs to cause oocyte maturation to progress.

Discussion

We have provided several lines of evidence that *Xenopus* P100 is an RNA binding component of cytoplasmic stored mRNPs. First, P100 was identified in complexes associated with FRGY2 and xRAP55A, both of which are components of stored mRNPs (Tanaka et al., 2006). Second, upon the fractionation of oocyte lysates with a sucrose gradient, P100 distributed predominantly in ribosome-free mRNP fractions, which indicates P100 is a component of stored and translationally repressed maternal mRNPs. Third, P100 was covalently bound to adenylated RNAs by UV-crosslinking. Finally, most of the known stored mRNP components were coimmunoprecipitated with anti-P100 antibody from stage I–III oocytes. Previously a protein of ~100-kDa, called mRNP6, was identified as a stored mRNP component in previtellogenic oocytes, although its molecular identity was not determined (Darnbrough and Ford, 1981; Murray et al., 1991). Our data suggest that mRNP6 might correspond to P100. We previously showed that the interaction of xRAP55A with FRGY2 is RNA-dependent in stage VI oocytes, while xRAP55A associates with Xp54 in a partly RNA-independent manner (Tanaka et al., 2006). The data presented in this paper together with a recent study by Minshall et al. (2007) demonstrate that the association of P100 with several proteins including Xp54, xRAP55A, xRAP55B, 4E-T and CPEB but not with FRGY2 is at least in part RNA-independent in small oocytes (Minshall et al., 2007). Transcription is highly active in small oocytes and a large pool of stored mRNPs is produced during this stage. Multiple translational repressors might associate with each other and function cooperatively to store newly synthesized mRNAs.

We found that P100 repressed translation of the reporter mRNA that was microinjected into oocytes. Translational repression was enhanced when P100 was tethered to the reporter mRNA. Thus our results with reporter mRNAs suggest that P100 functions as a translational repressor. In contrast, we found that P100 overexpression did not significantly affect global protein synthesis in oocytes, monitored by [^{35}S] methionine and cysteine incorporation. Overexpression of Pat1p in yeast leads to a global repression of translation and accumulation of mRNAs in P-bodies (Coller and Parker, 2005). Efficient translational repression and P-body assembly, which occur during glucose deprivation, require Pat1p. It is possible that, because a large proportion of maternal mRNAs are translationally repressed in oocytes and those mRNAs are already associated with endogenous P100, doubling the amount of P100 by injecting the *P100* mRNA would not have significantly affected global protein synthesis in oocytes. Alternatively, these differences between yeast Pat1p and *Xenopus* P100 on translation could be attributed to the low homology between them, one being a general repressor and the other not. There are two Pat1p homologues in vertebrates (Fig. 1A). Whereas P100 is an oocyte-specific protein, xPat1b started to accumulate in late oogenesis and was expressed during early development (Fig. S2). There seems to be a switchover from P100 to xPat1b during late oogenesis and oocyte maturation. Future work is necessary to clarify whether P100 and xPat1b, which are expressed differentially, are functionally equivalent.

Yeast Pat1p functions as a complex with Lsm1p-7p in activating mRNA decapping (Bonnerot et al., 2000; Bouveret et al., 2000; Tharun et al., 2000). Pat1p has five domains conserved among fungal species and through distinct domains associates with RNA and Dcp1p, Lsm1p and Edc3p, all of which are proteins involved in the decapping process (Pilkington and Parker, 2008). We found that P100 interacts with Lsm1 in *Xenopus* oocytes, thus presumably with the Lsm1–7 complex, because Lsm1 is the only unique component of the cytoplasmic Lsm1–7 complex. Although we do not know whether P100 promotes decapping, it is reported that there is no detectable decapping activity in *Xenopus* oocytes (Zhang et al., 1999). The absence of decapping activity in oocytes could make it possible that P100-tethered mRNAs are stored in a stable state and we could observe that P100 inhibits translation of reporter mRNAs (see below). Recently in *Caenorhabditis*

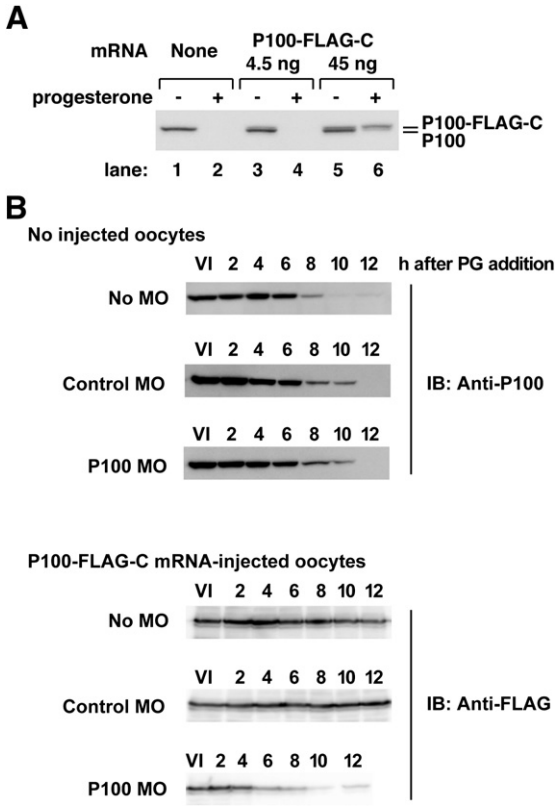

Fig. 6. Stability of P100 during oocyte maturation. (A) Oocytes uninjected (lanes 1 and 2) or injected with 4.5 ng (lanes 3 and 4) or 45 ng (lanes 5 and 6) of *P100-FLAG-C* mRNA were induced to mature with progesterone (lanes 2, 4 and 6). Lysates from stage VI and matured oocytes were analyzed by immunoblotting with anti-P100 antibody. (B) Stage VI oocytes were injected with 45 ng of *P100-FLAG-C* mRNA and incubated for 12 h. Oocytes were then injected with 20 ng of control MO or P100 MO. After an additional 12 h incubation, oocytes were induced to mature with progesterone. Every 2 h after progesterone addition, oocytes were harvested and analyzed for the endogenous P100 (top panels) or P100-FLAG-C (bottom panels).

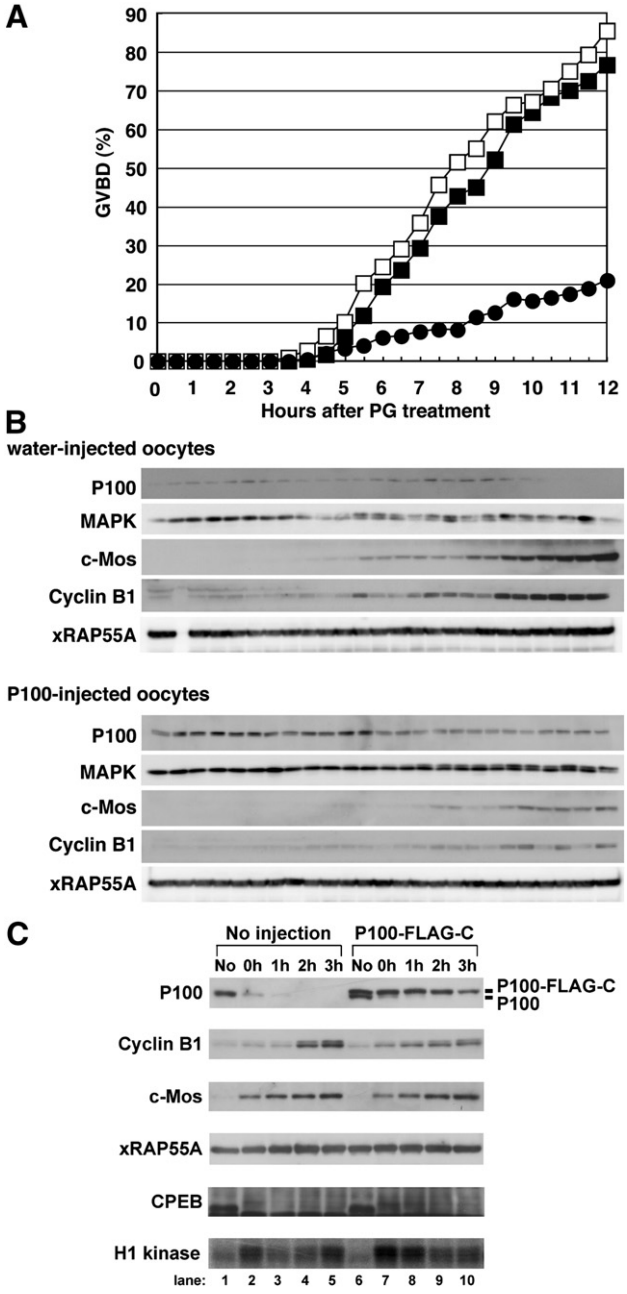

Fig. 7. P100 overexpression affects the accumulation of c-Mos and cyclin B1. (A–B) Oocytes were injected either with 45 ng of *P100-FLAG-C* mRNA (closed circles) or water (open squares), or not injected (closed squares). They were incubated for 12–14 h, and then stimulated with progesterone (PG). Oocytes were scored as a fraction of time for GVBD (A). Every 30 min after progesterone addition, oocytes injected with water or *P100-FLAG-C* mRNA were collected and analyzed by immunoblotting (B). The sample at 0.5 h of water-injected oocytes is missing in immunoblots for c-Mos, cyclin B1 and RAP55A. (C) Stage VI oocytes not expressing or expressing P100-FLAG-C were collected (lanes 1 and 6) or induced to mature with progesterone. The appearance of white spots was examined at 1-h intervals. At 8 h after the addition of progesterone, oocytes that underwent GVBD (0 h; lanes 2 and 7) were collected and those further incubated for 1 h (lanes 3 and 8), 2 h (lanes 4 and 9) and 3 h (lanes 5 and 10) after GVBD were then collected. Oocyte lysates were analyzed by immunoblotting or for histone H1 kinase activity.

elegans, PATR-1 was identified and characterized as a homologue of Pat1p. PATR-1 is expressed primarily in somatic tissues, where it is an integral component of mRNP granules (Boag et al., 2008; Gallo et al., 2008). A homozygous null mutant of PATR-1 produces offspring with arrested development during the late embryonic or early larval stages. Human Pat1b/PatL1 is localized to P-bodies in cultured cells and required for P-body formation (Scheller et al., 2007). Thus, the translational repression activity of Pat1p homologues appears to be conserved in various organisms.

P100 predominantly distributed to ribosome-free mRNP fractions when oocyte lysates were fractionated in a sucrose gradient. In this regard, P100 is an mRNP component that mostly, if not exclusively, associates with stored mRNAs. Given this, the finding that P100 disappears concomitant with GVBD raised the interesting possibility that P100 is involved in the storage of a class of mRNAs that are unmasked during oocyte maturation. Consistent with this, the accumulation of c-Mos and cyclin B1 was impaired in maturing oocytes that overexpressed P100. Further, we observed that P100-overexpressing oocytes undergo GVBD very poorly. These observations therefore suggest that P100 should disappear during oocyte maturation with normal kinetics and are consistent with the hypothesis that P100 represses translation of specific mRNA(s) such as *c-mos* and *cyclin B1* required for oocyte maturation.

Although it is yet unclear how P100 represses translation, the RNA binding affinity of Pat1p and the interaction of P100 with other proteins may explain how P100 is recruited to stored mRNPs. In *Xenopus* immature oocytes many mRNAs that will be unmasked during oocyte maturation are stored having short poly(A) tails (Mendez and Richter, 2001; Radford et al., 2008). The yeast Lsm1p–7p–Pat1p complex can associate with the 3′ end of oligo(A)-tailed mRNAs *in vivo* and *in vitro*, indicating that this complex has the intrinsic ability to distinguish between oligoadenylated and polyadenylated mRNAs (Tharun and Parker, 2001; Chowdhury et al., 2007). Thus, if one assumes that *Xenopus* Lsm1–7–P100 complex has a similar RNA binding affinity with that of yeast Lsm1p–7p–Pat1p complex, mRNAs that are stored in

oocytes with short poly(A) tails could be good substrates for the Lsm1–7–P100 complex. Further, many mRNAs that will be activated during oocyte maturation carry CPEs in their 3′UTRs (Richter, 2007; Radford et al., 2008). As P100 and CPEB interact with each other (at least in small oocytes) (Minshall et al., 2007 and this study), P100 and possibly Lsm1–7 complex could be recruited to CPE-carrying mRNAs preferentially. The timing of translational activation differs among CPE-carrying mRNAs: *c-mos* is translated earlier during oocyte maturation, prior to GVBD ('early class mRNA'), whereas *cyclin B1* is activated later, coincident with or after GVBD ('late class mRNA'). The timing depends on the combination and contexts of the CPEs and other cis-elements in 3′UTRs (Charlesworth et al., 2000, 2006; Nakahata et al., 2003; Wang et al., 2008; Pique et al., 2008; Belloc and Mendez, 2008). The disappearance of P100 may be required for the correct temporal translational activation of the late class mRNAs. One key future issue is to determine whether P100 represses cytoplasmic polyadenylation during oocyte maturation. Further functional analysis of protein complexes containing multiple mRNP components will shed light on these issues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.05.006.

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